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LPS-Induced iNOS mRNA and the Pro-Apoptotic Signaling Pathway  
in Leukocytes of Fit and Unfit Males

A thesis submitted in partial fulfillment of the requirements for the degree of Health and  
Movement Science, Master of Science, at Virginia Commonwealth University.

by

Tiffany M. Zúñiga

Bachelor of Science, Virginia Commonwealth University (VA), 2015

Director: Edmund O. Acevedo, Ph.D.

Professor, Associate Dean for Graduate Studies and Strategic Initiatives  
College of Humanities and Sciences

Virginia Commonwealth University  
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## Table of Contents

Abstract .....	iv
Chapter 1: Introduction.....	1
Specific Aims.....	3
Chapter 2: Literature Review .....	4
Innate Immune Function .....	4
Inducible Nitric Oxide Synthase .....	6
Aerobic Exercise.....	9
Chapter 3: Methodology.....	15
Chapter 4: Results.....	20
Chapter 5: Discussion .....	22
Table 1 .....	28
Figure 1 .....	29
Figure 2 .....	30
Figure 3 .....	31
Figure 4 .....	32
References .....	33
Curriculum Vitae .....	41

## Abstract

### LPS-INDUCED iNOS mRNA AND THE PRO-APOPTOTIC SIGNALING PATHWAY IN LEUKOCYTES OF HEALTH TRAINED MALES

By Tiffany M Zúñiga B.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Health and Movement Sciences for Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2018

Director: Edmund O. Acevedo, Ph.D.  
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Overexpression of the enzyme iNOS induces apoptotic cellular death by increasing indices of pro-inflammation and oxidative stress. Aerobic physical activity has been known to have anti-inflammatory benefits and reduce oxidative stress. **Purpose:** Therefore, this study aimed to examine the impact of aerobic fitness on LPS-induced iNOS mRNA expression and the relationship of this expression with indices of oxidative stress, pro-inflammation and apoptosis in isolated leukocytes. **Methods:** Whole blood samples from aerobically fit and unfit males were stimulated with and without LPS. Thereafter, iNOS mRNA expression and MDA, TNF- $\alpha$  and p53 concentrations were analyzed. **Results:** iNOS mRNA expression levels following LPS stimulation were not increased in both groups, and correlational analyses were not consistent with mechanistic predictions. **Discussion:** Numerous factors including timing of sample quantification, the high level of health of the subject population, and alternative intracellular

mechanisms impacting biomarkers analyzed, may have influenced leukocyte iNOS mRNA expression levels.

## Chapter 1: Introduction

Inducible nitric oxide synthase (iNOS) is an enzyme expressed in immunocompetent leukocytes that support innate immune function (Aktan, 2004; Niess et al., 2000; Niess et al., 2002; Soskic et al., 2011), and mediates the balance of reactive oxygen and nitrogen species (RONS) relative to antioxidant scavenging capacity that is associated with oxidative stress (Aktan, 2004; Assar et al., 2013; Bouzid et al., 2015; Curtin et al., 2002). Under normal resting conditions, iNOS expression is low-to-undetectable in leukocytes, and primarily aids in the production of nitric oxide (NO), a molecule typically involved in non-specific host defense and innate antioxidant function (Brune et al., 1998; Curtin et al., 2002; Kregel & Zhang, 2007; Lincoln et al., 1997). However, following cellular stimulation with the gram-negative bacterium lipopolysaccharide (LPS), activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factor enhances iNOS expression in concert with the synthesis of pro-inflammatory cytokines (Kim et al., 2004; Niess et al., 2000; Soskic et al., 2011). As a result, unregulated iNOS expression exacerbates the production of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF- $\alpha$ ), the accumulation of RONS, and subsequently, contributes to mitochondrial DNA damage and the induction of “programmed cell death” known as apoptosis (Assar et al., 2013; Craige et al., 2015; Curtin et al., 2002; Kannan & Jain, 2000; Kujoth et al., 2005; Ott et al., 2007; Phaneuf & Leeuwenburgh, 2001). Therefore, additional research focusing on the potential role of iNOS as a mediator of the apoptotic pathway in response to inflammatory challenge is warranted.

Aerobic exercise has been shown to regulate immune function (Blair et al., 2001; Gleeson et al., 2011; Kruger & Mooren, 2014; Mooren & Kruger, 2015). For example, chronic aerobic exercise training reduces systemic concentrations of pro-inflammatory cytokines and indices of oxidative stress at rest and in response to an acute bout of aerobic exercise (Bouzid et



al., 2015; Gliemann et al, 2013; Samjoo et al., 2013; Vinetti et al., 2015). Similarly, while leukocyte expression of iNOS mRNA is increased following a single bout of acute aerobic exercise, chronic exercise attenuates this response (Niess et al., 2000; Niess et al., 2002), potentially contributing to the decreased production of TNF- $\alpha$  and RONS in leukocytes following inflammatory challenge with LPS in individuals with enhanced aerobic fitness ( $VO_{2max}$ ) (Miyazaki et al., 2001). Although these findings suggest that enhanced aerobic fitness may differentially affect iNOS mRNA expression, no studies have investigated this relationship with TNF- $\alpha$  and RONS production, and apoptotic markers following *ex vivo* stimulation with LPS. Therefore, the primary aims of this study are to examine the LPS-induced expression of iNOS mRNA in isolated leukocytes in aerobically fit (high  $VO_{2max}$ ) and unfit (low  $VO_{2max}$ ) individuals. It is hypothesized that LPS-induced iNOS mRNA expression will be decreased in individuals with a higher  $VO_{2max}$ , and associated with the attenuated production of TNF- $\alpha$  and the oxidative stress marker lipid peroxidation marker (MDA) *ex vivo* at rest. Furthermore, to understand the potential role of iNOS in the maintenance of cellular homeostasis, this study will examine the relationship between LPS-induced iNOS mRNA expression and indices of apoptosis. It is hypothesized that lower iNOS mRNA expression in aerobically fit individuals will be associated with reduced concentration of the pro-apoptotic marker p53 following LPS-stimulation of leukocytes.

## Specific Aims:

- 1.) Examine the LPS-induced expression of iNOS mRNA in isolated leukocytes in aerobically fit (high  $\text{VO}_{2\text{max}}$ ) and unfit (low  $\text{VO}_{2\text{max}}$ ) individuals.

*Hypothesis:* LPS-induced iNOS mRNA expression will be decreased in aerobically fit (high  $\text{VO}_{2\text{max}}$ ) compared to unfit (low  $\text{VO}_{2\text{max}}$ ) individuals.

- 2.) Examine the relationship between LPS-induced iNOS mRNA expression with indices of pro-inflammation ( $\text{TNF-}\alpha$ ) and RONS (MDA).

*Hypothesis:* Lower iNOS mRNA expression will be associated with the attenuated production of  $\text{TNF-}\alpha$  and MDA *ex vivo* at rest.

- 3.) Examine the relationship between LPS-induced iNOS mRNA expression and the pro-apoptotic marker p53.

*Hypothesis:* Lower iNOS mRNA expression will be associated with reduced concentration of the pro-apoptotic marker p53 following LPS-stimulation of leukocytes.

## Chapter 2: Literature Review

### 2.1. Innate Immune Function

#### *2.1.1. The Innate Immune Response*

The innate immune system is a primary line of defense against infectious disease (Hoffmann et al., 1999; Janeway & Medzhitov, 2002). Circulating leukocytes, primarily monocytes and neutrophils, protect the host against systemic infection and foreign pathogens by initiating the innate immune response through the recognition of danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Aderem & Ulevitch, 2000; Hoffmann et al., 1999; Janeway & Medzhitov, 2002; Mogensen, 2009; Takeda et al., 2003; Takeda & Akira, 2005). This activation of PRRs on the cell surface of monocytes and neutrophils triggers the induction of intracellular signaling pathways responsible for increased gene expression and production of cytokines and chemokines that aggregate at the source of infection or pathogenic threat (Akira & Takeda, 2004). Thus, regulation of the leukocyte-mediated innate immune response is necessary for mediating cellular and systemic homeostasis.

#### *2.1.2. Inflammatory Signaling of Leukocytes Induced Oxidative Stress*

Toll-like receptor 4 (TLR4), a well-documented PRR expressed extracellularly on monocytes and neutrophils, is the primary mediator of the innate immune response following LPS stimulation (Akira & Takeda, 2004; Li & Verma, 2002). More specifically, the binding of LPS to TLR4 initiates the intracellular signaling pathways through activation of interleukin-1 receptor-associated kinase 4 (IRAK4) (Aderem & Ulevitch, 2000; Janeway & Medzhitov, 2002). Consequently, activated IRAK4 recruits and phosphorylates TNF-receptor-associated factor 6

(TRAF6), which is necessary for the activation of the inhibitor of kappa-B kinase (IKK) complex (Janeway & Medzhitov, 2002; Li & Verma, 2002). This signaling mechanism induces IKK to phosphorylate the inhibitor of kappa-B (I $\kappa$ B) from the NF- $\kappa$ B – I $\kappa$ B complex, (Li & Verma, 2002). Under resting conditions, I $\kappa$ B binds to NF- $\kappa$ B in the cytosol and inhibits the transcriptional activity of NF- $\kappa$ B within the nucleus (Li & Verma, 2002; Mogenson, 2009; Takeda & Akira, 2005). However, phosphorylation of I $\kappa$ B by IKK, releases NF- $\kappa$ B from I $\kappa$ B, resulting in the degradation of I $\kappa$ B and the nuclear translocation of NF- $\kappa$ B from the cytoplasm. Once translocated into the nucleus, NF- $\kappa$ B binds to DNA sites and up-regulates the transcription of pro-inflammatory cytokine genes, such as TNF- $\alpha$  (Akira & Takeda, 2004; Li & Verma, 2002; Mogensen, 2009; Takeda & Akira, 2005). Therefore, upon ligation to LPS, the TLR4-mediated inflammatory response results in the aggregation of inflammatory cytokines and proteins to the source of insult (Aderem & Ulevitch, 2000; Hoffmann et al., 1999; Janeway & Medzhitov, 2002; Takeda et al., 2003). However, persistent activation of the innate immune system through TLR4 results in the disruption of cellular homeostasis that contributes to chronic low-grade inflammation and disease pathology (Takeda & Akira, 2005).

Disruption of cellular homeostasis mediated through the LPS-TLR4-NF- $\kappa$ B signaling cascade also elicits a pro-oxidant effect and acts on mitochondrial respiration to produce intracellular RONS (Aktan, 2004). Furthermore, in a feed-forward cyclic mechanism, constant stimulation of TNF- $\alpha$  results in the persistent activation of the inflammatory pathway through the NF- $\kappa$ B mechanism. Interestingly, increased expression of iNOS has been shown to serve as a central mediator of LPS stimulated pro-inflammation and the production of intracellular RONS (Aktan, 2004; Aoi et al., 2010; Kormaz et al., 2009; Soskic et al., 2011). More specifically, elevated expression of iNOS is mediated by NF- $\kappa$ B and occurs in concert with the LPS-mediated

production of pro-inflammatory cytokines and chemokines (Morris & Billiar, 1994; Soskic et al., 2011). Given that iNOS has been shown to augment LPS-mediated production of TNF- $\alpha$  and ROS oxidative stress (Aktan, 2004; Niess et al., 2002; Soskic et al., 2011), it is noteworthy to suggest that additional examination in the regulation of iNOS following pro-inflammatory signaling may be necessary to further understand its role in regulating cellular homeostasis in leukocytes.

## **2.2. Inducible Nitric Oxide Synthase**

### **2.2.1. *iNOS***

Inducible nitric oxide synthase is expressed in a variety of cell types, such as astrocytes, microglial cells, neuronal cells, and leukocytes, and plays a central role in regulating innate immune function (Aktan, 2004; Niess et al., 2000; Niess et al., 2002; Reiling, et al. 1994; Soskic et al., 2011; Wolfe et al., 1994). Although iNOS expression is low in resting leukocytes, iNOS is a soluble enzyme that is elevated following stimulation by LPS and other pro-inflammatory stimulants, including TNF- $\alpha$  (Kim et. al, 2004; Morris & Billiar, 1994; Niess et al., 2000; Soskic et al., 2011). Therefore, most studies have reported the necessity for LPS stimulation to analyze iNOS expression in human cell types (Aktan, 2004; Niess et al., 2002; Niess et al., 1999 Soskic et al., 2011; Xie & Calycay, 1992). iNOS consists of two domains, one being the C-terminus that contains binding sites for NADPH and calmodulin (CaM) and an N-terminus that acts as an oxygenase domain and contains binding sites for heme and L-arginine (Morris & Billiar, 1994). When L-arginine binds, the iNOS enzyme can transform these arginases into NO at a level 100-1000 fold more than other NO synthases (Kormaz et al., 2009; Morris & Billiar, 1994). Furthermore, iNOS is calcium-independent and therefore does not require second messenger

reactions to occur in order to become stimulated and produce NO (Aktan, 2004). These findings suggest that iNOS assists in immune host defense by rapidly producing NO radicals within the leukocyte as a protective response against foreign pathogens (Bogdan, 2001). However, due to its stimulation through inflammatory mechanisms, iNOS activity can persist for hours after stimulation and continue to produce high levels of RONS (Morris & Billiar, 1994). Therefore, additional understanding regarding the role of iNOS-mediated NO, and the subsequent production of NO, is necessary to elucidate the mechanisms associated with cellular homeostasis.

### *2.2.2. iNOS Disrupts Cellular Homeostasis*

Oxidative stress is defined as an imbalance in the system between oxidant production and antioxidant defense that favor the former and can potentially lead to damaging effects within the cell (Davies et al., 1982; Sies, 1985; Sies 1997). Although naturally produced, RONS can be produced in elevated proportions during pathophysiological conditions, systemic inflammation, and through various stimuli including iNOS expression (Curtin et al., 2002; Sies, 1985; Sies, 1987). In addition, the continuous stimulation of iNOS results in the overproduction of NO and other indices of RONS, specifically  $\text{ONOO}^-$ . As a result, excess  $\text{ONOO}^-$  formation has been shown to contribute to cellular damage that can be measured through the free-radical oxidation reaction of polyunsaturated fatty acids that causes direct damage to lipids, also known as lipid peroxidation (Aktan, 2004; Ayala et al., 2014; Darley-USmar et al., 1995; Inoue et al., 2003; Gutteridge, 1995; Yoshida et al., 2013). This process involves three key steps including initiation, propagation, and termination (Ayala et al., 2014; Yin et al., 2011). First, the initiation of free radicals triggers a propagation of chain reactions, which ensue until termination products, such as malonaldehyde (MDA) and 4-hydroxynonenal are produced. These termination products

can then be measured as indices of elevated RONS production (Ayala et al., 2014; Yoshida et al., 2013), which in turn, initiate cellular death by apoptosis and contribute to the pathology of pro-inflammatory disease (Aktan, 2004; Luoma et al., 1998; Korkmaz et al., 2009).

Apoptosis is known as “programmed” cell death and is an important physiological mechanism that plays a key role in determining cellular development and homeostasis (Curtin et al., 2002; Kannan & Jain, 2000; Mukhopadhyay et al., 2014). Although leukocyte apoptosis is an important process that helps resolve the acute phase inflammatory process (Lawrence et al., 2001; Wesche et al., 2005), over-activation of the apoptotic pathway can lead to DNA fragmentation and mitochondrial damage (Curtin et al., 2002; Kannan & Jain, 2000; Phaneuf & Leeuwenburgh, 2001). Interestingly, iNOS has been shown to regulate the apoptotic pathway through the production of NO. More specifically, physiologically relevant concentrations of NO that are typically observed under resting conditions has anti-apoptotic effects within the cell by inhibiting several mechanisms associated with the apoptotic signaling pathway (Curtin et al., 2002). However, iNOS-induced overproduction of NO and subsequent ONOO<sup>-</sup> formation can activate apoptotic-signaling pathways (Curtin et al., 2002; Kannan & Jain, 2000; Nishiwaka et al., 1998).

The role of iNOS as a pro-apoptotic mediator was first recognized in the 1990's by various research groups who witnessed chromatic condensation and internucleosomal DNA fragmentation within the cell upon the overproduction of NO (Brune et al., 1998; Brune et al., 1999; MeBmer & Brune, 1996; Sandau et al., 1997). In addition, the activation of iNOS in macrophages led to NO toxicity (RONS) and the accumulation and stabilization of tumor suppressor p53, an initiator of apoptosis (Brune et al., 1999; MeBmer & Brune, 1996; Ott et al., 2007). Upon stabilization, p53 activity triggers the subsequent expression of Bcl-2 and Bax that

targets the mitochondria to release cytochrome c, which contributes to the downstream activation of pro-apoptotic caspases and cell death (Ott et al., 2007). Additionally, lipid peroxidation induced by iNOS-mediated NO production can also act on the mitochondrial membrane to release cytochrome c in concert with p53 activation (Korkmaz et al., 2009; Ott et al., 2007). These results suggest that iNOS and p53 may work synergistically to increase RONS production, induce apoptosis within the cell, and potentially, exacerbate the pro-inflammatory response (Korkmaz et al., 2009; Ott et al., 2007). Furthermore, cellular stimulation with LPS and the pro-inflammatory cytokine TNF- $\alpha$  induces apoptosis by activating pro-apoptotic proteins and causing a downstream cascade that eventually leads to cell death (Wesche et al., 2005). Therefore, while these findings suggest that pro-inflammatory stimulation of leukocytes, and the concomitant induction of the apoptotic pathway, would be mediated through the elevated expression levels of iNOS, no studies have investigated this hypothesis. Similarly, further research is warranted to understand if interventions such as chronic aerobic exercise may be utilized as a method of regulating these mechanisms and cellular health.

## **2.3. Aerobic Exercise**

### *2.3.1. Aerobic Exercise Enhances Immune Function*

It has been established that chronic engagement in physical activity and exercise can lower the risk of age-related diseases and support a healthy immune system (Blair et al., 2001). After an acute bout of exercise a local inflammatory response occurs that involves the production of cytokines that are released at sites of inflammation and the influx of lymphocytes and monocytes (Gleeson, 2007; Pederson & Hoffman-Goetz, 2000). However, chronic exercise has been shown to have significant anti-inflammatory effects to mediate this acute phase response



(Gleeson et al., 2011). Chronic aerobic exercise inhibits monocyte infiltration into adipose tissue, reduces circulating monocytes with a pro-inflammatory phenotype, and stimulates the increase of anti-inflammatory cytokines (Gleeson et al., 2011). Given that iNOS may be a key mediator in these immune responses, it is important to investigate how exercise may modulate iNOS expression within the leukocytes and determine how this may change the response mechanisms in response to the engagement of physical activity designed to increase levels of aerobic fitness.

### *2.3.2. iNOS and Acute Exercise*

iNOS expression has been shown to be altered with both acute and chronic aerobic exercise. It has previously been demonstrated that a single bout of intense, endurance exercise induces expression of iNOS in leukocytes at a transcriptional level (Niess et al., 2000). More specifically, running at 110% of an individual's anaerobic threshold for approximately 17.7 minutes increases leukocyte iNOS protein expression, whereas runners who participated in a half marathon exhibited an increased expression of iNOS mRNA in leukocytes immediately following and 3 hours into recovery from exercise (Niess et al., 2000). These results demonstrate that the elevated iNOS mRNA and protein expression within leukocytes is dependent upon both the duration and intensity of strenuous exercise (Niess et al., 2000). Furthermore, this could be due to the acute phase stimulation of pro-inflammatory cytokines such as TNF- $\alpha$  and the increased number of circulating leukocytes observed following a bout of strenuous exercise (Huang et al., 2011b; Gleeson, 2007). Although the regulatory pathways leading to exercise-induced expression of iNOS in human leukocytes are not well documented, it is still plausible that several activators, such as TNF- $\alpha$ , modulate iNOS expression through the NF- $\kappa$ B signaling pathway (Aktan, 2004; Kormaz et al., 2009). Furthermore, flow cytometric results demonstrate

an increased baseline expression of iNOS in leukocytes, with the greatest expression observed within monocytes (Niess et al., 2000), suggesting that iNOS expression in monocytes is necessary to regulate the innate immune system during stressful physiological conditions. Acute aerobic exercise has a direct effect on apoptosis and its ability to regulate the apoptotic pathway during conditions such as oxidative stress and inflammation (Kruger & Mooren, 2014; Ott et al., 2007). Currently, there is evidence that shows that strenuous exercise stimulates apoptosis to occur in both skeletal muscle and lymphocytes (Kruger & Mooren, 2009; Mooren & Kruger, 2014; Phanuef & Leeuwenburgh, 2001). Additionally, RONS and TNF- $\alpha$  are both elevated after an acute bout of exercise, and have the potential to induce apoptosis through the signaling mechanisms previously described (Kormaz et al., 2009; Gleeson, 2007). Thus, the elevated levels of TNF- $\alpha$  observed following acute aerobic exercise might propagate the apoptotic response through iNOS stimulation and the subsequent production of RONS (Kormaz et al., 2009).

Previous evidence also suggests that a single bout of exhaustive exercise results in augmented levels of macromolecule damage mechanisms, including lipid peroxidation (Radak et al., 2001; Radak et al., 2008). Previous research has indicated an increase in oxidative stress related markers (Hunag et al., 2010a). More specifically, multiple studies have reported a significant increase in MDA levels following maximal or near maximal exercise protocols ( $\geq 70\% \text{VO}_{2\text{max}}$ ) (Bailey et al., 2004; Bryant et al., 2003; Fatouros et al., 2001; Goldfarb et al., 2007; Kanter et al., 1993). While these studies suggest that exercise-induced oxidative damage does occur at certain exercise intensities and modes, it may be plausible that the elevated production of NO and ONOO<sup>-</sup>, lipid peroxidation, and ultimately, cell death, results from overexpression of iNOS.

### 2.3.3. *iNOS and Chronic Exercise*

iNOS expression is also modulated with chronic endurance exercise training. For example, Niess et al. (2002) demonstrated that iNOS mRNA expression levels were lower in moderately trained endurance runners compared to their untrained counterparts, potentially due to the attenuated release of pro-inflammatory cytokines observed in the trained subjects (Niess et al., 2000). Similarly, regular exercise attenuated iNOS mRNA expression and the production of RONS products in colon cancer patients (Aoi et al., 2010). Although the mechanisms associated with this response were not directly investigated, the reduced production of RONS may have been the result of more physiologically relevant NO concentrations that help positively regulate cellular homeostasis. Interestingly, chronic exercise also helps attenuate the elevated expression of iNOS mRNA typically observed in response to acute exercise in both rodents and human models (Erekat et al., 2013; Harris et al., 2008; Vassilakopoulos et al., 2003). These findings suggest that chronic aerobic exercise training may be beneficial to cellular homeostasis by regulating iNOS expression.

Training status has an inverse effect on exercise-induced apoptosis in lymphocytes. For example, Mooren et al. (2004) demonstrates that cell death only occurred in those that were considered less trained compared to those who were well trained. Similarly, Peters et al. (2006) observed prolonged exercise (running at 75%  $\text{VO}_{2\text{max}}$  for 2.5 hours), which typically induces cellular apoptosis in sedentary individuals, had no effect in well-trained athletes. In both human and mice models, similar data suggests that there was no change in apoptotic markers after an acute bout of exercise with regular exercise training (Kruger & Mooren, 2014). This could be indicative of positive adaptations or regulation of the immune system that reduces the activation of the apoptotic pathway that is induced by iNOS. However, there is limited literature describing

the effect of chronic exercise on iNOS-induced apoptosis. Therefore, research is warranted to understand how chronic exercise may play a key role in suppressing iNOS expression as a mechanistic link to attenuate cellular apoptosis, and thus, regulate the innate immune response within the leukocytes.

It has been demonstrated that if a trained individual performs the same exhaustive exercise as their sedentary counterpart, the system adapts to attenuate the elevated response of radical production that may occur (Huang et al., 2013; Miyazaki et al., 2001; Radak et al., 2008; Radak et al., 2013). Therefore, exercise-induced adaptations in trained individuals are able to modulate RONS handling and have oxidative damage repair systems that untrained individuals may not experience (Radak et al., 2013). In a study conducted by Miyazaki et al. (2001) individuals endured chronic endurance training that proved to attenuate indices of oxidative stress and suggested that this reduction improved RONS handling. Additionally, it would be expected that regular exercise training would attenuate RONS productions and shift the redox balance in favor of reducing conditions that cause cellular damage (Radak et al., 2008; Urso & Clarkson, 2003). Therefore, it may be beneficial to investigate if the decreased iNOS expression seen in trained or aerobically fit individuals is associated with the attenuated RONS production found in previous studies.

Although it has been demonstrated that endurance training can attenuate iNOS expression in multiple cell types, no research has examined the impact of aerobic fitness on iNOS mRNA expression following pro-inflammatory challenge within leukocytes. Furthermore, the effect of chronic aerobic exercise and the iNOS-induced apoptotic pathway has yet to be examined. Therefore, research regarding this signaling mechanism is warranted to understand the possible

role of exercise in regulating this mechanism to enhance the innate immune function of cells in humans.

## **2.4. Conclusion**

iNOS is an inflammatory enzyme that may have a key role in regulating immune function. Stimulated by LPS and pro-inflammatory cytokine release, overexpression of iNOS causes on overproduction of NO through the LPS-TLR4-NF- $\kappa$ B signaling cascade. The constant overproduction of NO leads to indices of RONS, such as the formation of ONOO<sup>-</sup>, and can cause lipid peroxidation and DNA damage within the cell. Until this signaling mechanism is dampened or resolved, damage to the cells macromolecules will proceed and result in cellular death by apoptosis. However, chronic aerobic exercise participation has been shown to support a healthy immune system and counter regulate the pro-inflammatory signaling pathway. Therefore, given that regular participation in aerobic exercise has been shown to reduce iNOS mRNA expression within leukocytes, an examination of the relationship of iNOS mRNA expression with the LPS-induced immune response and activation of the pro-apoptotic pathway is warranted. Results from such studies will provide beneficial insight regarding the role of iNOS in mediating cellular homeostasis.

## Chapter 3: Methodology

### 3.1. Subject Participation

Thirty healthy male subjects (15 aerobically fit, 15 aerobically unfit) between the ages of 18-35 years were recruited to participate in this study. Subjects were excluded if identified as tobacco users, consumers of ten or more standard alcoholic drinks per week, or having any pre-existing cardiovascular, pulmonary, or metabolic diseases known to alter immune function. Prior to study, each subject completed an informed consent form and medical history questionnaire. In addition, each subject completed a seven-day physical activity recall. Subjects who participated in  $< 150$  minutes of moderate-to-vigorous physical activity, including aerobic, anaerobic, or resistance type exercise, and a  $VO_{2max} < 50 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  (detailed below) were classified as *aerobically unfit*. To the contrary, subjects who participated in  $\geq 150$  minutes of moderate-to-vigorous aerobic (i.e., running, cycling, and rowing) and a  $VO_{2max} \geq 55 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  were classified as *aerobically fit* (Slusher et al., 2018). Furthermore, subjects were instructed to partake in an overnight fast for approximately eight hours and to refrain from alcohol consumption, caffeine intake, and intense physical activity for at least 24 hours prior to their participation in the study. Of note, our laboratory's previous research indicates the cellular responsiveness to LPS stimulation returns to baseline levels within 1 hour following participation in acute physical activity (Slusher et al., 2017), suggesting that the 24 hour period withdrawing from physical activity is sufficient to prevent any residual impact on the results from the present study. This study was approved by the University's Institutional Review Board.

### *3.2. Exercise Testing Session*

Subjects were asked to arrive at the laboratory at 6:00 a.m. on the morning of testing sessions. Prior to exercise, subject's resting heart rate, blood pressure, and anthropometric measures height, weight, waist, and hip circumference were measured. Additionally, each individual was familiarized with instrumentation and procedures. Thereafter, a maximal oxygen consumption ( $\text{VO}_{2\text{max}}$ ) test was administered on a treadmill to reach maximal exertion.

$\text{VO}_{2\text{max}}$  was determined using ParvoMedics Metabolic Measurement System (ParvoMedics, Sandy, UT, USA). Heart rate was assessed and recorded by heart rate monitors (Polar T31, Polar Electro, Kempele, Finland) prior to, during exercise, and in recovery. Rate of perceived exertion, using Borg 20-point Scale was recorded every exercise stage. During stage 1, the treadmill's speed was adjusted to elicit a heart rate that is 85% of subject's age-predicted maximal heart rate. During stage 2, the subject continued at the same speed for two minutes at a 1% incline to reach a steady state. Thereafter, the incline was increased by a 2% grade every two-minutes until volitional exhaustion was reached or test termination. Criteria for attaining a  $\text{VO}_{2\text{max}}$  included the obtainment of three of more of the following criteria as detailed by the American College of Sports Medicine guidelines (ACSM, 2013):  $\text{RER} \geq 1.15$ , heart rate within 10bpm of subject's age predicted maximum heart rate,  $\text{RPE} \geq 19$ , and blood lactate  $\geq 8\text{mmol}$ .

### *3.3. LPS-stimulation of Whole Blood*

Two whole blood samples (6 mL) were collected at rest by venous puncture from the antecubital vein using a 21G butterfly needle into a tube containing  $\text{K}_2$  ethylenediaminetetraacetic acid ( $\text{K}_2\text{EDTA}$ ) (BD Vacutainer, Franklin Lakes, NJ). Upon

collection, one whole blood tube was stimulated with 10 ng/mL of LPS (Abbasi et al., 2013) (Liang et al., 2013; Slusher et al., 2018) and the other whole blood tube remained unstimulated and served as a time-course control. Each whole blood sample was incubated at 37°C with 5% CO<sub>2</sub> for three hours with manual inversion every fifteen minutes. Immediately after incubation with inversions, whole blood samples were centrifuged at 3000 rpm for twenty minutes at room temperature to obtain the plasma supernatant, which was subsequently centrifuged at 10,000×g for ten minutes at 4°C to obtain platelet-free plasma. Following the centrifuge, aliquots of plasma were transferred to freshly labeled tubes and stored at -80°C for later analysis of TNF- $\alpha$  (R&D Systems, Minneapolis, MN, USA), MDA (Northwest Life Science Specialties, Vancouver, WA, USA) and p53 (ThermoFisher Scientific, Frederick MD) in duplicate by enzyme-linked immunosorbent assay methods according to manufacturer's instructions.

### *3.4. RNA Isolation and cDNA Synthesis*

Whole blood (2.5 mL) containing the leukocyte cell layer was isolated from both the LPS-stimulated and unstimulated samples and transferred into PAXGene Blood RNA tubes (PreAnalytix, Switzerland) and inverted manually 8-10 times. Samples were stored at room temperature for two hours, transferred to -20°C for overnight storage, and transferred again to -80°C for long-term storage according to manufacturer's instructions. Total RNA isolation, using PAXGene Blood RNA kit according to the manufacturer protocol, provided for quantitative real-time polymerase chain reaction (qPCR) analysis (PreAnalytix, Switzerland). The concentration of extracted RNA was then measured spectrophotometrically by Nanodrop 1000 (Thermo Scientific, Waltham, MA). Only samples with A<sub>260/280</sub> ratios above 1.7 were used for cDNA synthesis. 1000ng of total RNA was used as a template for cDNA synthesis using (Quanta BioSciences, Beverly, MA) cDNA synthesis kit according the manufacturer's protocol.



### 3.5. Quantitative real-time PCR

A 20ng sample of RNA was reversed transcribed (5 minutes at 22°C, 30 minutes at 42°C, 5 minutes at 85°C, followed by a 4°C hold) to examine relative expression of iNOS using qPCR. Reverse transcription (RT) and polymerase chain reaction (PCR) master mix consisted of 7.5uL of SYBR green, 2uL of nuclease-free water, 0.75uL each of sense and antisense primers, and 4uL of cDNA sample totaling 15uL per reaction mix. For the amplification of desired iNOS cDNA, the following gene specific primers were used: iNOS-sense 5'-CAGCGGGATGACTTTCCAA-3', iNOS antisense 5'-AGGCAAGATTTGGACCTGCA-3',  $\beta$ -actin sense 5'-AGCGGGAAATCGTGCGTG-3' and  $\beta$ -actin antisense 5'-CAGGGTACATGGTGGTGCC-3'. The PCR reactions were performed in triples to ensure accuracy. Additionally, qPCR was carried out by an automated DNA thermal cycler located at the laboratory and melting curve analysis was generated after 45 rounds of thermal cycling. iNOS relative expression was analyzed using  $2^{-\Delta\Delta C_t}$  method. To ensure further accuracy of measurement, 25% of samples were run additional times. No differences were observed within these results.

### 3.6. Statistical Analysis

Statistical data was analyzed using SPSS software. Independent samples t-test were utilized to assess anthropometric and cardiorespiratory fitness in aerobically fit versus unfit individuals. Additionally, a 2 group (aerobically fit and unfit) by 2 condition (unstimulated time-course control and LPS stimulated) repeated measures analysis of variance (RMANOVA) was utilized to examine the interaction between aerobic capacity and iNOS expression, MDA

concentration, and p53 expression. Independent samples t-tests were conducted to assess differences between groups within LPS-stimulated markers. Paired samples t-tests were utilized to assess differences across culture conditions within each group. Pearson's correlation was used to determine relationship between iNOS expression and percent changes of MDA, TNF- $\alpha$ , and p53 as well as associations presented between relative VO<sub>2max</sub> and our markers.

## Chapter 4: Results

### 4.1. Subject Characteristics

Subject descriptive statistics are presented in Table 1. Although no differences in anthropometric measures were observed between aerobically fit and unfit subjects, aerobically fit individuals presented with lower resting HR values ( $t_{[22.877]} = -3.816, p = 0.001$ ) and greater absolute and relative  $VO_{2max}$  values compared to aerobically unfit subjects ( $t_{[28]} = 7.431, p \leq 0.001$ ;  $t_{[28]} = 11.437, p \leq 0.001$ , respectively). These observed differences suggest that subsequent findings are the result of improved cardiovascular health and cardiorespiratory fitness in aerobically fit compared to unfit subjects.

### 4.2. iNOS mRNA expression and MDA, TNF- $\alpha$ , and p53 concentrations following ex vivo stimulation with LPS

Repeated measures ANOVA (RMANOVA) revealed that LPS stimulation did not increase iNOS mRNA expression in aerobically fit or unfit counterparts relative to the unstimulated time-course control (*Condition Effect*:  $F_{[1,26]} = 75.952, p = 0.146$ ; Figure 2A). In addition, no differences in the constitutive (unstimulated control) release of MDA, TNF- $\alpha$ , or p53 were observed among aerobically fit and unfit subjects as determined from the unstimulated culture control conditions. However, RMANOVA revealed that LPS stimulation significantly lowered MDA concentrations to a greater extent in aerobically unfit compared to fit subjects (*Group \* Condition Effect*:  $F_{[1, 28]} = 15.137, p = 0.001$ ; Figure 2B), whereas LPS stimulation increased TNF- $\alpha$  and lowered p53 to a similar extent in both groups (*Condition Effect*:  $F_{[1, 28]} = 12.087, p = 0.002$ ;  $F_{[1, 28]} = 5.868, p = 0.022$ , respectively; Figures 2C and D).

#### *4.3. Associations among relative $VO_{2max}$ , LPS stimulated iNOS mRNA expression, and MDA, TNF- $\alpha$ , and p53 concentrations*

Consistent with the aforementioned results, relative expression of iNOS mRNA following LPS-stimulation was not significantly correlated to relative  $VO_{2max}$  ( $r = -0.121$ ,  $p = 0.263$ ; Figure 3A). In addition, while the percent change in the production of MDA from the unstimulated control to the LPS stimulated culture condition was positively associated with relative  $VO_{2max}$  ( $r = 0.558$ ,  $p = 0.001$ ; Figure 3B), no significant associations were observed between TNF- $\alpha$  and p53 with relative  $VO_{2max}$  ( $r = -0.104$ ,  $p = 0.628$ ;  $r = -0.069$ ,  $p = 0.717$ , respectively; Figures 3C and D). Finally, change in relative iNOS mRNA expression was not associated with the percent change in the production of MDA, TNF- $\alpha$ , and p53 from the unstimulated control to the LPS stimulated culture condition ( $r = -0.059$ ,  $p = 0.757$ ;  $r = -0.368$ ,  $p = 0.076$ ;  $r = -0.132$ ,  $p = 0.487$ , respectively; Figure 4A-C).

## Chapter 5: Discussion

This study sought to investigate the potential role of increased aerobic fitness levels on the LPS-induced expression of iNOS mRNA and its association with indices of oxidative stress, pro-inflammation, and apoptosis. Results from this report demonstrated that iNOS mRNA expression was not differentially expressed following LPS stimulation of whole blood in aerobically fit and unfit individuals. Additionally, while LPS stimulation decreased MDA concentrations to a greater extent in aerobically unfit compared to fit subjects, both groups responded with increased and decreased concentrations of TNF- $\alpha$  and p53, respectively. Finally, these responses were not associated with relative iNOS mRNA expression, suggesting that a change in iNOS mRNA expression in response to LPS stimulation does not significantly influence MDA, TNF- $\alpha$ , or p53 concentrations within this subject population.

Transcriptional regulation of iNOS is mediated through the NF- $\kappa$ B pro-inflammatory signaling pathway (Akira & Takeda, 2004; Li & Verma, 2002; Mogensen, 2009; Takeda & Akira, 2005), and elevated expression levels of iNOS mRNA have been observed in rodent models of diseased populations where NF- $\kappa$ B is typically over activated (Akita et al., 2007; Fujimoto et al., 2005; Miyoshi et al., 2006; Soskic et al., 2011). In humans, iNOS mRNA expression has also been shown to be elevated in middle-aged populations with pro-inflammatory conditions, such as rheumatoid arthritis, compared to healthy controls (age 51-61) (St. Clair et al., 1996). Similarly, *in vitro* stimulation of isolated monocytes with LPS (1  $\mu$ g/mL) for five days has been shown to elicit a two-fold increase in iNOS mRNA expression in individuals with rheumatoid arthritis, whereas no iNOS activity was observed in healthy controls (St. Clair et al., 1996). Furthermore, Schena et al. (1999) revealed that although iNOS mRNA expression was undetectable in monocytes of young, normotensive individuals, significant

increases were observed in response to stimulation with epinephrine, dopamine, and endothelin 1. Interestingly, regular physical activity and exercise training have been shown to elicit an anti-inflammatory response through several mechanisms, including the inhibition of pro-inflammatory cytokine activity that is known to counteract an LPS-induced inflammatory response (Bruunsgaard, 2005). Therefore, we investigated the impact of LPS stimulation on iNOS mRNA expression and whether or not enhanced aerobic fitness also downregulates this response. Our results demonstrated that LPS-stimulation was insufficient to significantly increase iNOS mRNA expression in healthy, aerobically unfit and fit individuals. A potential explanation for these results may be that the protective effects of physical activity, which were hypothesized to be mediated by an attenuated expression of iNOS mRNA, become more apparent with disease and increased age, and therefore, were not observable due to the positive health and relatively young age of the subjects in the present study. Likewise, it has also been demonstrated that the iNOS promoter contains a regulatory component that binds NF- $\kappa$ B repressing factor, a constitutively expressed silencer of iNOS, and suppresses basal expression at the transcriptional level (Korhonen et al., 2005). Therefore, it may be that the low to undetectable expression levels of iNOS mRNA within unstimulated cells indicates a mechanism that is responsible for the instability of iNOS mRNA in young, healthy individuals under resting conditions (De Vera et al., 1996; Korhonen et al., 2005).

The impact of regular participation in aerobic exercise on iNOS mRNA expression remains unclear. For example, various studies utilizing rodent models have demonstrated that chronic wheel-running exercise increases basal iNOS mRNA expression in macrophages, endothelial cells, and cardiac myocytes compared to wheel-restricted counterparts (Lu et al., 1994; Yang et al., 2002; Akita et al., 2007). However, such investigations in human tissue and

innate immune cells have produced inconsistent results. More specifically, Niess et al. (1999) have shown that iNOS mRNA expression within unstimulated leukocytes were low or undetectable in trained subjects compared to their untrained counterparts, suggesting that aerobic exercise training attenuates constitutive iNOS expression under resting, physiological conditions. To the contrary, Su et al. (2011) demonstrated that exercise training in young, healthy populations significantly increased basal iNOS mRNA expression in neutrophils, but not in lymphocytes or monocytes. Likewise, elevations in iNOS were still observed up to four weeks after a detraining period and negatively associated with indices of neutrophil apoptosis (Su et al., 2011), suggesting that the impact of aerobic exercise training on basal iNOS mRNA expression in humans may be cell specific.

The lack of iNOS mRNA expression changes observed in the present study may have also been the result of the culture conditions utilized, including LPS concentration and the duration of *ex vivo* stimulation period. More specifically, the present study incubated whole blood samples with 10 ng/mL of LPS for 3 hours. However, several other groups demonstrated that iNOS protein expression significantly increased following stimulation with LPS concentrations ranging from 5-15,000 ng/mL and incubation periods of 3-8 hours, with peak protein concentration being observed after 6 hours (Ambrozova et al., 2010; Ambrozova et al., 2011; Palazzolo-Balance et al., 2007; Pekarova et al., 2009). Therefore, it is possible that the shorter incubation period during LPS stimulation may not have been long enough to elicit a robust response and affected the significance of our results. Furthermore, it has been previously documented that high LPS concentrations increases intracellular calcium, thereby inhibiting iNOS expression (Korhonen et al., 2001; Korhonen et al., 2005). Therefore, it is also possible that the supra-physiological concentrations of LPS utilized in the present study (nearly 1000

times greater than those previously reported by Selkirk et al. [2008] in a similar population of young aerobically fit and unfit males) initiated a calcium-dependent signaling mechanism which defended the overexpression of iNOS mRNA within innate immune cells of relatively healthy males.

The present study also observed an expected increase in TNF- $\alpha$  concentrations following LPS-stimulation in both aerobically fit and unfit subjects (Aderem & Ulevitch, 2000; Hoffmann et al., 1999; Janeway & Medzhitov, 2002; Takeda et al., 2003), and this response tended to be negatively associated with iNOS mRNA expression ( $r = -0.368$ ,  $p = 0.076$ ). TNF- $\alpha$  concentrations released from monocytes following stimulation with LPS peak within 4 hours, much more rapidly than iNOS (Takashiba et al., 1999), and increased TNF- $\alpha$  concentrations have previously been shown to activate the second messenger protein, cyclic adenosine monophosphate (cAMP), which serves as a negative feedback mechanism to inhibit NF- $\kappa$ B activation and prevent the overactivation of the pro-inflammatory signaling pathways. Furthermore, cAMP has been shown to inhibit iNOS mRNA stability within specific cell subsets and potentially attenuate iNOS mRNA expression upon increased TNF- $\alpha$  concentrations (Korhonen et al., 2005). Therefore, the increased concentrations of TNF- $\alpha$  released from stimulated immune cells may have initiated a secondary signaling mechanism that prevented the increased expression of iNOS mRNA in the present study (Galea & Feinstein, 1999; Satriano & Schlondorff, 1994).

This investigation also investigated the impact of LPS stimulation on the MDA and p53 concentrations. Surprisingly, although MDA concentrations were not significantly different between subject groups in the unstimulated samples, MDA concentrations significantly decreased to a greater extent in the aerobically unfit compared to fit group following LPS



stimulation. These results were similar with Djordjevic et al. (2012), who also observed a significant decrease in the pro-oxidant marker thiobarbituric acid reactive substance in young, healthy, non-athletes following a maximal exercise test. Although it is plausible that individuals with decreased aerobic fitness levels exhibited a more robust antioxidant response to combat increased levels of oxidative stress, the lack of investigation into this point is a limitation of the present study, and future studies would benefit from the assessment of resting antioxidant levels to accurately determine if participation in chronic aerobic exercise influenced regulatory mechanisms associated with oxidative stress and any combative influence antioxidants may have in concert with iNOS expression. Furthermore, our results did show a significant decrease in p53 concentration upon LPS stimulation, independent of iNOS mRNA expression. This is inconsistent with previous literature that displayed an increase in p53 accumulation upon inflammatory stimulation (Meßmer & Brune, 1996; Meßmer and Brune, 1997). However, a recent study in rodents determined that p53 also decreased in response to LPS-stimulation (3,000 unit/g body weight) of endothelial cells (Barabutis et al., 2015). Investigators suggested a reciprocal negative relationship between p53 and the NF- $\kappa$ B-mediated production of the inflammatory cytokine interleukin 6, which downregulate p53 transcriptional activity as a consequential anti-inflammatory response necessary to counter regulate the LPS induced pro-inflammatory effects (Barabutis et al., 2015). Although this was determined in a different cell population, it is plausible that a similar mechanism is occurring within the leukocytes of healthy males as an adaptive response to inflammatory insult.

In conclusion, this investigation determined that healthy individuals with increased aerobic fitness levels did not significantly exhibit differences in iNOS mRNA expression following LPS stimulation, and consequently, maintenance of iNOS mRNA expression did not

directly impact indices of oxidative stress, pro-inflammation, or the apoptotic marker p53.

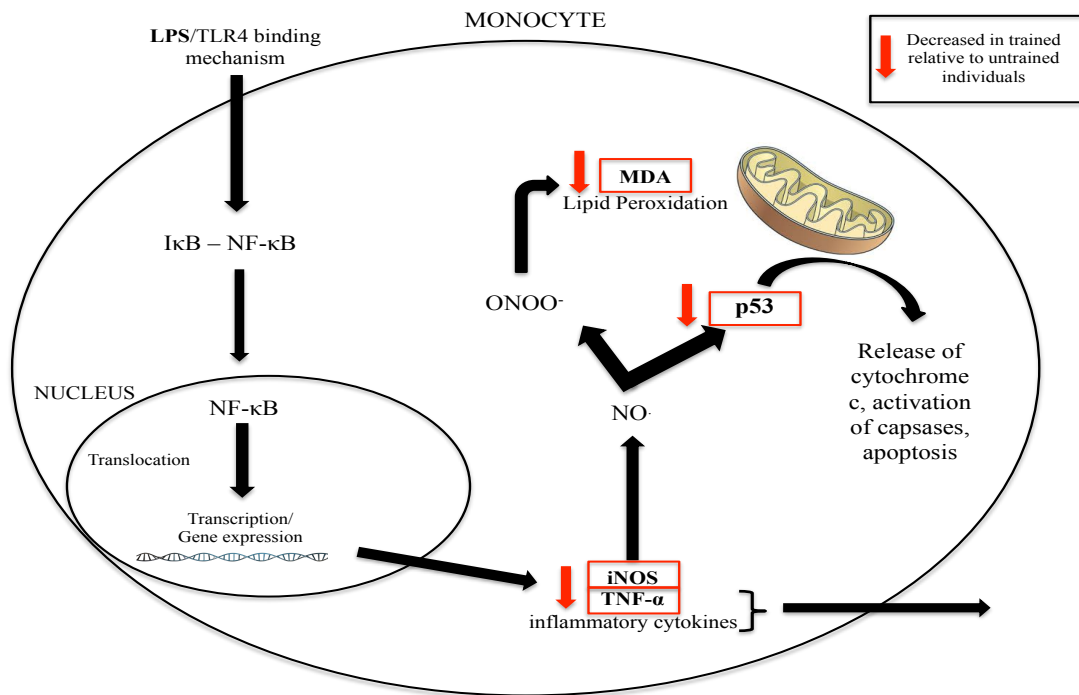
Nonetheless, it is apparent that iNOS mRNA stability is impacted by a variety of intracellular mechanisms and findings from the present study provoke the need for subsequent investigations to understand the appropriate stimulation necessary to elucidate a potential change in iNOS mRNA expression within a young, healthy subject population. Such studies would help to explain the beneficial regulatory innate immune response within leukocytes, and in particular, monocytes.

**Table 1. Subject Characteristics****Table 1.** Subject anthropometric characteristics, cardiovascular measures, and indices of cardiorespiratory fitness

Variable	Trained (n = 15)	Untrained (n = 15)	<i>P</i> value
Age (y)	25.27 ± 4.574	23.13 ± 3.502	0.163
Weight (kg)	69.420 ± 6.347	70.273 ± 8.709	0.761
Height (m)	1.790 ± 0.052	1.778 ± 0.082	0.621
BMI (kg/m <sup>2</sup> )	21.681 ± 1.975	22.185 ± 1.744	0.465
Waist (cm)	75.493 ± 3.098	77.333 ± 5.093	0.676
Hip (cm)	95.407 ± 3.461	96.740 ± 5.795	0.451
W:H	0.791 ± 0.027	0.780 ± 0.035	0.454
Body Fat Percentage	11.080 ± 4.096	13.440 ± 0.035	0.163
Resting HR (bpm)	60.200 ± 5.348	70.467 ± 8.943	0.001*
Resting SBP (mmHg)	117.333 ± 8.269	113.333 ± 8.997	0.215
Resting DBP (mmHg)	78.400 ± 9.891	77.067 ± 8.581	0.696
Absolute VO <sub>2max</sub> (L·min <sup>-1</sup> )	4.367 ± 0.406	3.145 ± 0.491	< 0.001*
Relative VO <sub>2max</sub> (mL·kg <sup>-1</sup> ·min <sup>-1</sup> )	63.047 ± 4.290	44.793 ± 4.450	< 0.001*

**Note:** The \* indicates a significant difference between trained and untrained subjects at baseline ( $p < 0.05$ ). Data are presented as means ± S.D. BMI, body mass index; W:H, waist-to-hip ratio; HR, Heart Rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; VO<sub>2max</sub>, maximal oxygen uptake

**Figure 1. LPS-induced iNOS Activation Signaling Mechanism**



**Figure 2.**

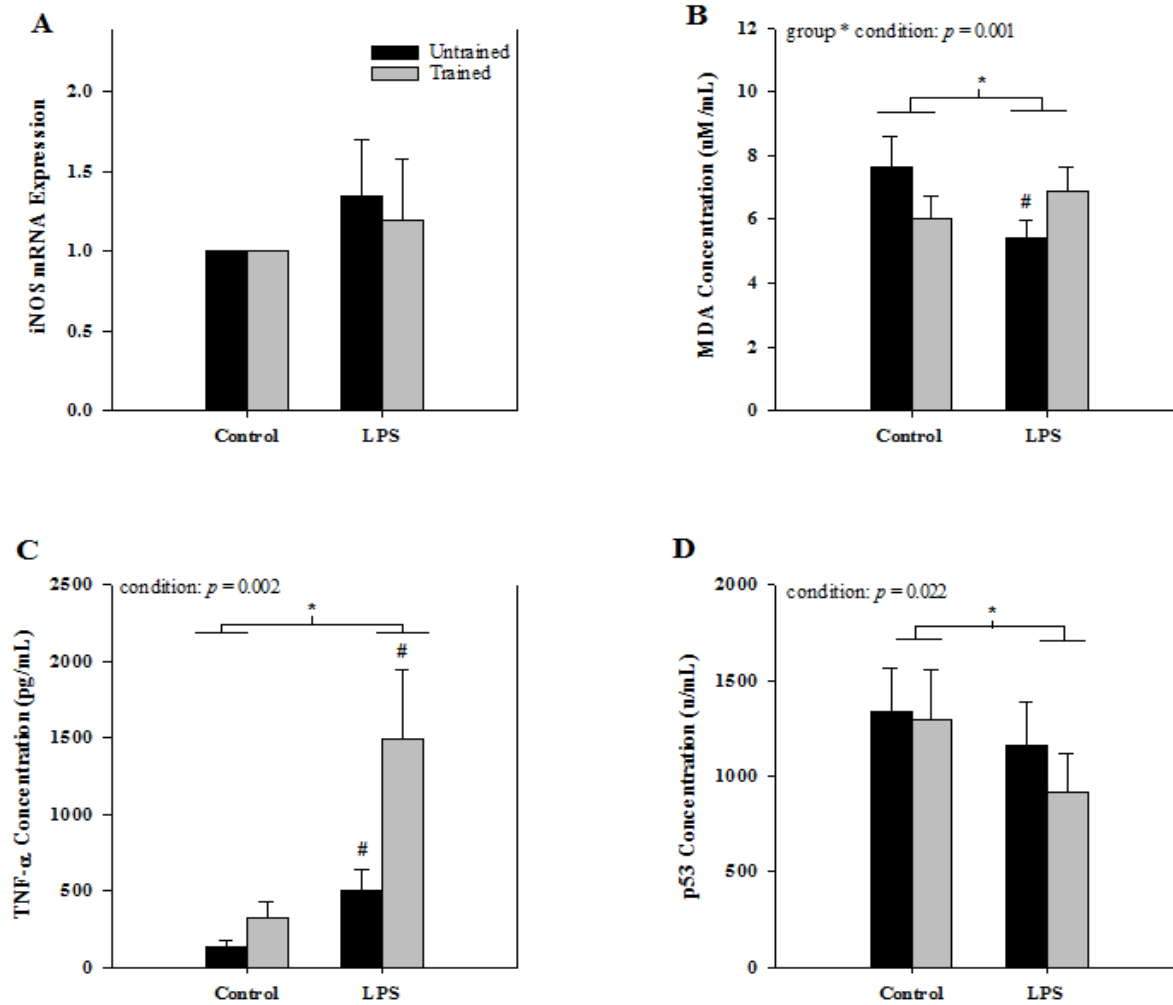


Figure 2. iNOS mRNA expression and concentrations of the lipid peroxidation marker MDA, the pro-inflammatory cytokines TNF- $\alpha$ , and pro-apoptotic marker p53 following ex vivo stimulation with LPS (Panels A-D). \* indicates a significant difference in the LPS-stimulated response relative to the unstimulated control in all subjects grouped; the # indicates a significant difference in LPS-stimulated MDA production relative to the unstimulated control within each group ( $p \leq 0.05$ ).

Figure 3.

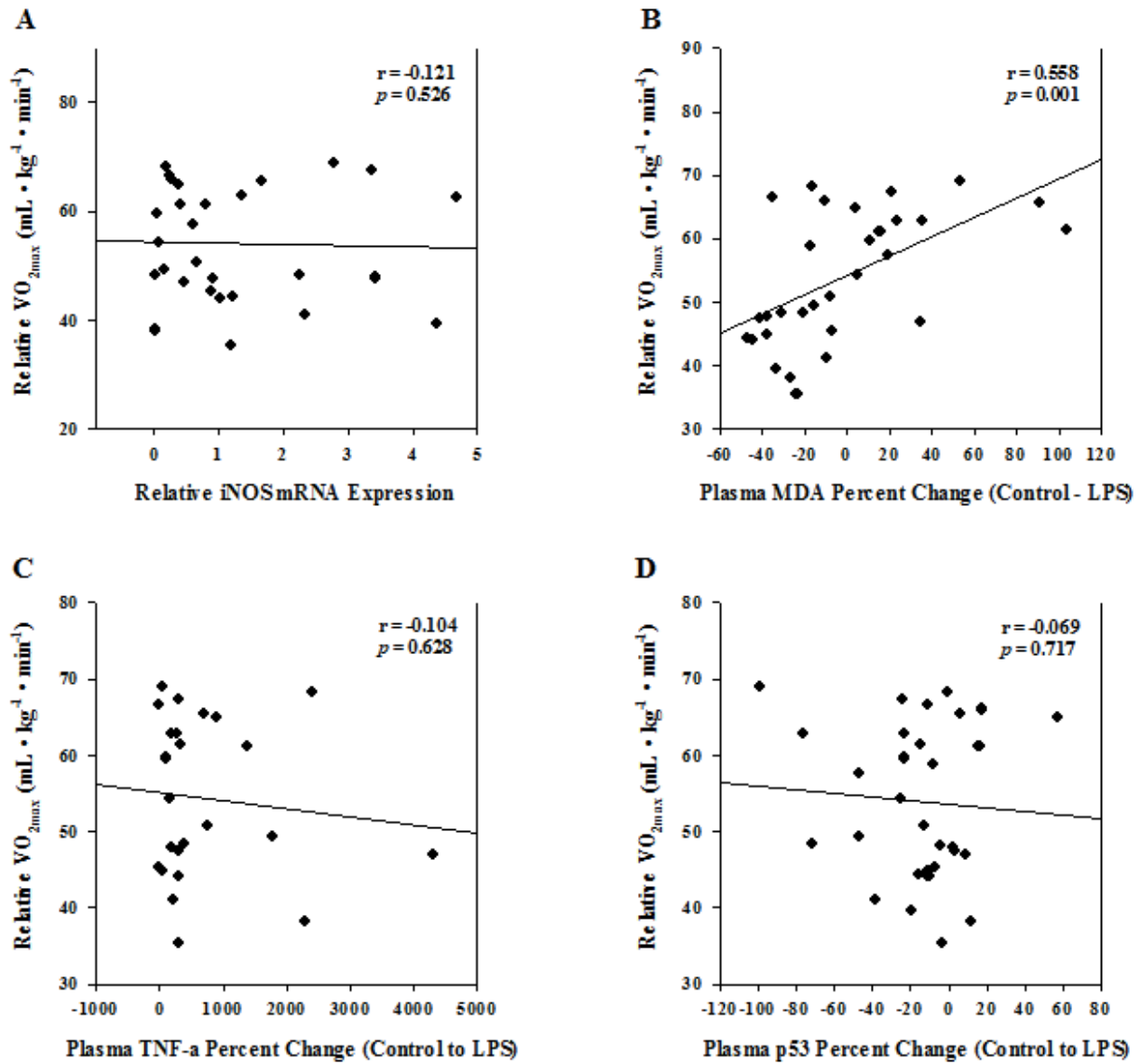


Figure 3. The association of relative  $VO_{2max}$  with relative iNOS mRNA expression, and concentrations of the lipid peroxidation marker MDA, the pro-inflammatory cytokines TNF- $\alpha$ , and pro-apoptotic marker p53 following ex vivo stimulation with LPS (Panels A-D).

**Figure 4.**

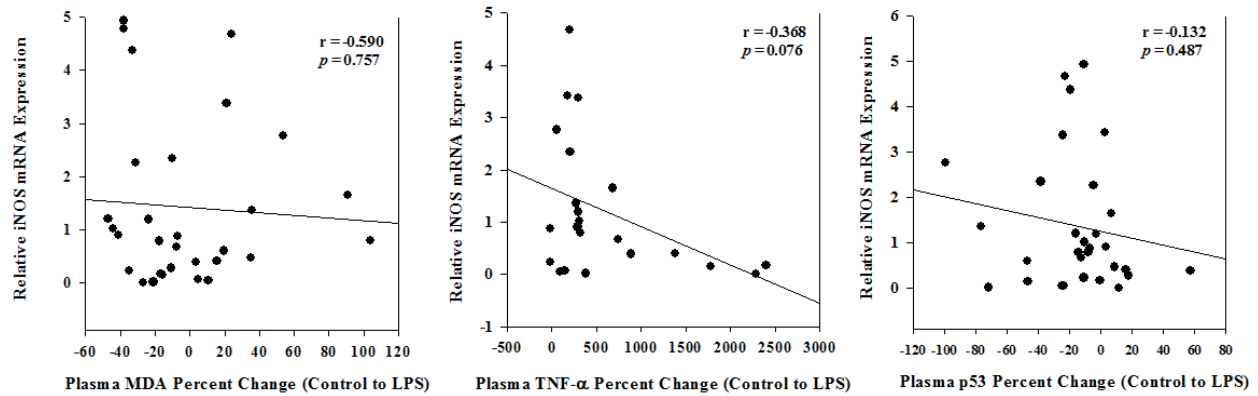


Figure 4. The association of relative iNOS mRNA expression with concentrations of the lipid peroxidation marker MDA, the pro-inflammatory cytokines TNF- $\alpha$ , and pro-apoptotic marker p53 following ex vivo stimulation with LPS (Panels A-C). *a.*) The association between relative iNOS mRNA expression and plasma MDA percent change from the unstimulated control to the LPS stimulated culture condition. *b.*) The tended negative association between relative iNOS mRNA expression and plasma TNF- $\alpha$  percent change from unstimulated control to the LPS stimulated culture condition. *c.*) The association between relative iNOS mRNA expression and plasma p53 percent change from unstimulated control to LPS stimulated culture condition.

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## Curriculum Vitae

Tiffany Marie Zúñiga was born on July 7<sup>th</sup>, 1993 in Las Vegas, Nevada. She attended Centreville High School, graduating class of 2011, in Centreville, Virginia. Her education continued at Virginia Commonwealth University where she has completed her Bachelor of Science and is working towards her Master of Science. She hopes to complete her doctoral degree at the University of Arizona in Tucson. Below is her current and most up to date accomplishments/achievements, and body of work that she has completed thus far in her research career.

### PRESENT POSITION

Graduate Research Assistant	Address:	1020 W. Grace Street
Department of Kinesiology and Health Sciences		Richmond, VA 23284
College of Humanities and Sciences	Phone:	571-266-9563
Virginia Commonwealth University	Email:	zunigatm@vcu.edu

### EDUCATION

#### 2016-Current Master of Science

Virginia Commonwealth University, Richmond, VA  
Kinesiology and Health Sciences: Health and Movement Science  
**Thesis Title: LPS-induced iNOS mRNA and the Pro-Apoptotic Signaling Pathway in Leukocytes of Healthy Trained Males**  
Thesis Committee Members: Dr. Edmund O. Acevedo, PhD, FACSM, FAPA, FNKA (Chair)  
Dr. Lee Franco, PhD, ACSM EP-C  
Dr. Ryan S. Garten, PhD

**2011-15 Bachelor of Science: *Magna Cum Lauda***  
Virginia Commonwealth University, Richmond, VA  
Health, Physical Education, and Exercise Science: Exercise Science

### PROFESSIONAL EXPERIENCE

#### 2016-Current Graduate Research & Teaching Assistant

Department of Kinesiology and Health Sciences  
Virginia Commonwealth University  
Richmond, VA

### TEACHING EXPERIENCE

#### 2017-Current HPEZ 375 Physiology of Exercise Lab

Virginia Commonwealth University  
Department of Kinesiology and Health Sciences



Undergraduate Course

1section– 1 credit

**2017-Current HPEX 250 Medical Terminology**

Virginia Commonwealth University

Department of Kinesiology and Health Sciences

Undergraduate Course

1section– 1 credit

**PROFESSIONAL AFFILIATIONS**

**2016-Current American College of Sports Medicine**

2016 National American College of Sports Medicine Conference, Boston, MA

2017 National American College of Sports Medicine Conference, Denver CO

**2015-Current Southeast Region American College of Sports Medicine**

2018 Southeast Regional American College of Sports Medicine Conference,  
Chattanooga, TN

2017 Southeast Regional American College of Sports Medicine Conference,  
Greenville, SC

2016 Southeast Regional American College of Sports Medicine Conference,  
Greenville, SC

2015 Southeast Regional American College of Sports Medicine Conference,  
Jacksonville, FL

**PEER REVIEWED SCIENTIFIC PUBLICATIONS**

1. Slusher AL, **Zúñiga TM**, Acevedo EO. Maximal Exercise Alters the Inflammatory Phenotype and Response of Mononuclear Cells. *Med Sci Sports Exerc.* 2018 (*Epub ahead of print*)

**SCIENTIFIC MANUSCRIPTS UNDER REVIEW**

1. Slusher AL, **Zúñiga TM**, Acevedo EO. Aerobic Fitness Alters the Capacity of Mononuclear Cells to Produce Pentraxin 3 Following Maximal Exercise.
2. Garten RS, Scott MC, **Zúñiga TM**, Hogwood AC, Fralin RC, Weggen JB. Lipid Peroxidation is Unaltered Following a Three Hour Bout of Prolonged Sitting.

**SCIENTIFIC MANUSCRIPTS IN PROGRESS**

1. **Zúñiga TM**, Slusher AL, Acevedo EO. LPS-induced iNOS mRNA and the Pro-Apoptotic Signaling Pathway in Leukocytes of Healthy Trained Males.
2. Slusher AL, **Zúñiga TM**, Acevedo EO. Impact of Maximal Exercise on the Anti-Inflammatory Capacity of Pentraxin 3 in Trained and Untrained Males
3. Garten RS, Scott MC, **Zúñiga TM**, Hogwood AC. The Effect of High Intensity Interval Exercise on Prolonged Sitting-Induced Vascular Dysfunction.

## SIGNIFICANT SCIENTIFIC RESEARCH CONTRIBUTIONS

### Acknowledgments

1. Slusher AL, Mischo AB, Acevedo EO. Pentraxin 3 is an anti-inflammatory protein associated with lipid-induced interleukin 10 in vitro. *Cytokine*. 2016;86;36-40.

### POSTER PRESENTATIONS

1. **Zúñiga TM**, Scott MC, Hogwood AC, Garten RS. The Effect of High Intensity Interval Exercise on Prolonged-Sitting Induced Vascular Dysfunction. Southeast Region College of Sports Medicine Annual Meeting (2017), Greenville SC.
2. Garten RS, **Zúñiga TM**, Scott, MC, Hogwood AC. Prolonged-Sitting Induced Lower Limb Vascular Dysfunction: The Effect of Oxidative Stress. College of Sports Medicine Annual Meeting (2017), Denver, CO.
3. Slusher AL, Mischo AB, **Zúñiga TM**, Acevedo EO. Maximal Exercise Alters the Inflammatory Phenotype of Mononuclear Cells and Response to *Ex Vivo* LPS Stimulation. Southeast Region College of Sports Medicine Annual Meeting (2017), Greenville SC.  
*\*\*Nominated for the 2017 SEACSM Doctoral Poster Research Award*
4. Slusher AL, Mischo AB, **Zúñiga TM**, Acevedo EO. Maximal Exercise Alters the Inflammatory Phenotype of Mononuclear Cells and Response to *Ex Vivo* LPS Stimulation. American College of Sports Medicine Annual Meeting (2017), Denver, CO. – *Immunology Section, May 31, 2017* *\*\*Oral Presentation: Immunology Section May 31, 2017*
5. Slusher AL, Mischo AB, **Zúñiga TM**, Acevedo EO. How Does Acute Aerobic Exercise Alter Innate Immune Function? Southeast Region American College of Sports Medicine Annual Meeting (2017), Greenville SC. *\*\* Oral Presentation: Pre-Conference Tutorial II, Rapid Research Race Presentation February 16, 2017*
6. **Zúñiga TM**, Slusher AL, Acevedo EO. Aerobic Training Status and Fatty-Acid Induced hTERT mRNA Expression Following Maximal Exercise. Southeast Region College of Sports Medicine Annual Meeting (2018), Chattanooga, TN.
7. Slusher AL., **Zúñiga TM**, Acevedo EO. Aerobic Fitness Alters the Capacity of Mononuclear Cells to Produce Pentraxin 3 Following Maximal Exercise. Southeast Region College of Sports Medicine Annual Meeting (2018), Chattanooga, TN.
8. Scott MC, Hogwood A, Fralin R, Lee D, Weggen J, **Zúñiga TM**, Garten RS. The Impact of Sleep Quality on Peripheral Vascular Function in Young Adults. Southeast Region College of Sports Medicine Annual Meeting (2018), Chattanooga, TN.

## LABORATORY EXPERIENCE AND TECHNIQUES

### Current Research

**2016-2017      Research Assistant**

NIH R21 Research Grant

Subjective Responses and Metabolic State During Exercise in Sickle Cell Anemia

Principal Investigator: Dr. Suzanne Ameringer PhD, RN

**Applied Exercise Physiology Testing**

Anthropometric Assessment

Maximal Oxygen Consumption Testing

Submaximal Aerobic Testing

Lactate and Anaerobic Threshold

Resting Metabolic Rate

**Biochemical Techniques**

Enzyme-linked Immunosorbent Assay

Human Peripheral Mononuclear Cell Culturing

Human Peripheral Mononuclear Cell Isolation

Phlebotomy

Protein Extraction

RNA Isolation

cDNA Synthesis

Quantitative Real-Time Polymerase Chain Reaction

**Ultra-Sonography**

Flow Mediated Dilation

Passive Leg Movement